

CHANGES OF ANTIOXIDANT ENZYME ACTIVITY AND HEAT SHOCK PROTEIN CONTENT IN LYMPHOCYTES OF CHILDREN WITH ASTHMA

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Abstract — The aim of the present study was to examine whether changes in the antioxidant status and expression of major intracellular stress proteins are associated with pathophysiology of childhood asthma and severity of the disease. The activities of copper/zinc (CuZn) and manganese (Mn) superoxide dismutase (SOD) and catalase (CAT) in peripheral blood mononuclear cells (PBMCs) of 28 stable pediatric patients (11 mild persistent and 17 moderate persistent asthmatics) and 12 healthy children were assessed by native gel assays. The levels of two heat shock proteins (HSPs), Hsp70 and Hsp90, were examined by quantitative Western blot analysis. Moderate asthmatics, in comparison to healthy children, displayed higher activity of CuZnSOD, while differences of MnSOD and CAT activity between the groups of patients were not found. The levels of Hsp90 and the inducible isoform of Hsp70 were increased in moderate asthmatics as compared to mild asthmatics and healthy children. The results demonstrate an imbalance in cellular antioxidant and stress response systems that may contribute to pathogenesis of childhood asthma, but are not necessarily related to severity of the disease.

Key words: Asthma, children, superoxide dismutase, catalase, heat shock proteins

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INTRODUCTION

Asthma is a chronic pulmonary disorder characterized by airway inflammation. Many experimental and clinical data suggest that oxidative stress is increased in asthmatic children and adults (Kirkham and Rahman, 2006; Vachier et al., 1994; Shanmugasundaram et al., 2001; Liao et al., 2004; Mak et al., 2004). Impairment of the antioxidant system and/or excessive production of reactive oxygen species by inflammatory cells, which overwhelms host antioxidant defenses, are well recognized mechanisms of molecular and cellular damage and dysfunction in the airways. These mechanisms are believed to contribute to the pathophysiology of asthma.

To minimize the harmful effects of an oxidative burden, cells are endowed with a variety of defense mechanisms. Antioxidant enzymes constitute the main protection against free radical-mediated inju-

ry. The bulk of reactive species detoxification is achieved through a two-step pathway in which the superoxide anion ($O_2^{\cdot -}$) is converted to another oxidant [hydrogen peroxide (H_2O_2)] by superoxide dismutase (SOD), and H_2O_2 is chemically neutralized by catalase (CAT). There are two intracellular SOD isoforms in mammals: cytoplasmic copper/zinc SOD (CuZnSOD) and mitochondrial manganese SOD (MnSOD). In regard to asthma, these enzymes are believed to play a significant role against oxidative stress in lung (Kinnula and Carpo, 2003; Comhair et al., 2005; Smith et al., 1997; Gnosh et al., 2006) and blood (Shanmugasundaram et al., 2001; Mak et al., 2004; Pennings et al., 1999) cells, but also in plasma or serum (Liao et al., 2004), which are predominantly investigated in pediatric patients.

As the most studied and best understood effectors of the cellular stress response, heat shock proteins (HSPs) are also recognized as major com-

ponents of the cytoprotective system. Among many members of this large protein family, Hsp70 and Hsp90 are designated as key molecular chaperones participating in folding and maturation of multiple components of signal transduction pathways. Protein folding and denaturation are particularly enhanced during various stressful conditions, including oxidative stress. The findings that Hsp70 and Hsp90 are modulated by oxidizing conditions (Polla et al., 1996; Ménoret et al., 2002; Nardai et al., 2000) and by antioxidant supplements (Peng et al., 2000) support the regulatory role of oxidant/antioxidant balance in heat shock protein expression (Calabrese et al., 2006). HSPs generally perform a protective function, but can also amplify an inflammatory response. These proteins, particularly Hsp70, have a capacity to stimulate expression of proinflammatory cytokines and induce antigen-specific immunity (Pockley et al., 2003). A recent study has shown that Hsp70 overexpression is correlated with upregulation of HLA-DR surface molecules in bronchoalveolar lavage (BAL) cells and bronchial biopsies of asthmatics, giving rise to the hypothesis that Hsp70 has a potential role in antigen processing and/or presentation (Bertorelli et al., 1998). Despite their still unresolved (protective, amplificatory, or dual) role in inflammation and asthma (Polla et al., 1998), the elevated expression of HSPs is considered to be indicative of an enhanced cellular stress response. Moreover, the data on their potential impact on childhood asthma are scarce.

In light of the importance of antioxidative defense mechanisms and the heat shock response for cellular defense against oxidative stress, and hence, for the pathogenesis of asthma, the purpose of this study was to compare the activities of key antioxidant enzymes (CAT, SODs) and levels of HSPs (Hsp70, Hsp90) in asthmatic and healthy children. Being performed on subjects suffering from mild and moderate asthma, the study is designed to provide new information on possible association of the studied enzyme activities and expression of the HSPs with asthma severity. To conduct this study, circulating blood mononuclear cells were used since they play a prominent role in the inflammatory pro-

cess and have been shown to contribute to elevated oxidative stress in asthma.

MATERIALS AND METHODS

Patients

A total of 40 children were enrolled in the study. They were recruited from the outpatient clinic of the Children's Hospital for Tuberculosis and Pulmonary Diseases. The population consisted of 28 children with two different degrees of asthma severity (11 with mild persistent asthma and 17 with moderate persistent asthma) and 12 age-matched healthy controls attending the clinic for unrelated conditions. All patients with asthma were diagnosed by well-known criteria, that is, clinical symptoms and lung function measurements according to Global Strategy for Asthma Management (NIH publication no. 02-3659, Bethesda, 2002). Based on a history of physician-diagnosed asthma and medication used, patients with FEV1 of $\geq 80\%$ of predicted variability or peak expiratory flow (PEF) variability between 20 and 30% combined with occasional symptoms were classified as mild persistent asthmatics, while patients with FEV1 of 60 to 80% of predicted variability or PEF variability of 30% with daily symptoms were classified as moderate persistent asthmatics. Eligibility criteria included a documented asthma diagnosis during the last 12 months or longer, and age between 8 and 18 years. Exclusion criteria included: (1) other chronic lung disease, (2) treatment with oral or inhaled corticosteroids during the last four weeks, (3) treatment with antihistamines and theophylline during the last two weeks, (4) treatment with β_2 -agonists during the last 48 hours and (5) asthma exacerbations during the last 8 weeks. For control patients, the eligibility criteria included normal lung function, good health, and age between 8 and 18 years, while the exclusion criteria were diagnosis of asthma and upper or lower respiratory tract infection during the last 4 weeks. Blood drawing appointments were set if and when the patient fulfilled the inclusion criteria and none of the exclusion ones.

Atopic status was assessed by the skin-prick test for common allergens (feathers, home dust,

mould, dust mites, cockroaches, tobacco, animal hair, *Dermatophagoides pteronyssinus*, bacteria, tree pollen, grass pollen, weed pollen, *Ambrosia artemisiifolia*). Physiological solution and histamine served as controls.

The children and their parents were informed of the purpose of the study and gave their consent. The Ethics Committee of the Dr. Dragiša Mišević Clinical Center approved the study protocol.

Blood sampling and cell isolation

After the patient was made comfortable, blood was obtained by venipuncture in heparinized tubes (25 U/ml) between 8:30 and 9:30 a.m. The samples were immediately taken to the laboratory and cells were isolated within one hour from blood drawing.

To isolate peripheral blood mononuclear cells (PBMCs), blood was diluted with an equal volume of phosphate buffer saline (PBS) (0.14 mmol/l NaCl, 2.7 mmol/l KCl, 1.5 mmol/l KH_2PO_4 , 6.5 mmol/l Na_2HPO_4 ; pH 7.2) and subjected to density gradient centrifugation over Ficoll-Hypaque Plus (Amersham Biosciences) at 380xg for 30 min at 23 °C. The cells were collected, washed two times with PBS and the pellet was stored in liquid nitrogen until analysis.

Cellular protein extraction

For protein extraction, cellular pellets were resuspended in ice-cold hypotonic lysis buffer (50 mmol/l NaCl, 5% glycerol, 0.5% Triton X-100, 2 mmol/l ethylenediaminetetraacetic acid and 50 mmol/l Tris; pH 7.5) supplemented with a complete protease inhibitor cocktail (Roche Diagnostics GmbH) using 15 µl of extraction buffer per 1×10^6 cells. Following incubation on ice for 15 min and two freeze/thaw cycles, the lysate was centrifuged at 13,000xg for 15 min at 4 °C. The supernatants, referred to as cell extracts, were stored in small portions at -75°C. Protein concentration, estimated using bovine serum albumin as standard, ranged from 3 to 6 µg/µl.

Measurement of SOD and CAT enzyme activities

Cell extracts were diluted 1:1 with native sample buffer (10% glycerol, 0.0075% bromphenol blue

and 62.5 µmol/l Tris; pH 6.8) and non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed using a Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA). Lanes were loaded with 100 µg of protein and electrophoresis was run at a constant voltage of 120 V through 1 mm-thick 11% polyacrylamide (PA) gel (30% T, 2.7% C_{bis}) in Tris-glycine running buffer (pH 8.3). SOD activity was detected in the gel by the modified method of Beauchamp and Fridovich (1971). The gel was incubated in the dark in a solution containing 0.5 mmol/l nitroblue tetrazolium (NBT; Sigma Chemical Co., USA) and 50 mmol/l potassium phosphate (pH 7.8) for 15 min, followed by a 15 min soak in 28 µmol/l riboflavin and 28 mmol/l tetramethylethylenediamine (TEMED; Sigma Chemical Co., USA) solution. Subsequently, the gel was illuminated on a light box for 25 min until clear SOD bands appeared on a dark purple background. The isoforms were identified by adding 5 mM H_2O_2 and 5 mM KCN to buffered NBT solutions, CuZnSOD being sensitive and MnSOD unaffected by the treatment (data not shown).

For analysis of CAT activity, 1 mm-thick 6% PA gel was loaded with 100 µg of total protein and native PAGE was performed under the conditions as described above. The gel was thoroughly washed with dH_2O and after 10 min of incubation in 0.007% H_2O_2 was quickly rinsed with dH_2O . A freshly prepared mixture of 2% ferric chloride and 2% potassium ferricyanide was poured over the gel. When clear bands of CAT activity appeared on a green background, the reaction was stopped by replacing the staining mixture with 7% acetic acid and a 5% methanol solution. Stained gels were scanned with an Ultra-Lum GEL EXPLORER Imaging System (Claremont, CA).

Western blot analysis of heat shock proteins

Cell extracts were reduced and denatured by 5-min treatment at 95 °C with buffer containing 62.5 µmol/l Tris (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.0075% bromphenol blue. Proteins (70 µg per lane) were resolved on 7.5% SDS-PA gel, together with protein molecular mass standards (29-205 kDa; Sigma Chemical Co., USA).

After overnight immunoblotting, PVDF membranes (Hybond-P, Amersham Biosciences) were incubated in blocking buffer containing 2.5% nonfat dry milk in PBS (pH 7.2) for 1.5 h at room temperature and then probed with an appropriate primary antibody. The primary antibodies used were: SPA-830, raised against mouse Hsp90 (Stressgen Bioreagents, Victoria, BC, Canada; working dilution of 1:2,000); SPA-820 (Stressgen Bioreagents; 1:2,000) which recognizes both constitutive (Hsc70) and inducible (Hsp72) isoforms of Hsp70; SPA-810, which is specific for Hsp72 (Stressgen Bioreagents; 1:500); and anti- β -actin (Sigma Chemical Co., USA; 1:8,000). After incubation with primary antibodies and three cycles of washing with PBS containing 0.1% Tween 20, the membranes were incubated with alkaline phosphatase-conjugated secondary antibody for 1 h. The immunoreactive proteins were visualized on STORM (Amersham Pharmacia Biotech UK Limited, England) by the enhanced chemifluorescent method.

Quantitative analysis of Western blots and gels

Each lane was loaded with an equal amount of protein, the β -actin bands serving as the equal load control for Western blot analysis. To make quantitative comparisons between multiple immunoblots or enzyme assays reliable, i.e., to counterbalance inter-gel variations, an internal reference sample (cellular extract from PBMCs donated by a healthy adult female volunteer) was run on each gel. Prior to any comparison, the intensity of each analyzed band was normalized to the intensity of the respective internal reference band on the same blot or gel. Relative integrated optical density of immunoreactive bands was measured using Image Quant software (Amersham Biosciences Limited, UK) and the results are expressed in arbitrary units (AU).

Statistical analysis

Nonparametric tests were used to analyze the data. Differences between groups are presented as mean differences with a 95% confidence interval. The Kruskal-Wallis test, with a subsequent *post hoc* test, was performed for comparison of the three groups of patients. Between-group comparisons

were assessed by the Mann-Whitney U-test. A two-tailed *p* value of less than 0.05 was considered significant. Statistical comparisons were carried out using the GraphPad Prism v4.0 software (GraphPad Prism Software Inc., San Diego, CA).

RESULTS

Healthy children and children with mild asthma were similar ($p=0.3$) in terms of age, 16.1 ± 0.5 years (mean \pm SE, $n=12$, range 13-18) and 14.6 ± 0.9 years (mean \pm SE, $n=11$, range 9-18), respectively. The average age of children with moderate asthma was 13.7 ± 0.6 years (mean \pm SE, $n=17$, range 8-17), and they were significantly younger ($p=0.01$) than the healthy subjects. The male to female ratio was six to six in the healthy group of children, six to five in the group of mild asthmatics, and 14 to three in the group of moderate asthmatic children. Duration of the disease was similar ($p=0.1$) in both groups of asthmatic children: 7.8 ± 1.4 years (mean \pm SE, range 1-13.5) in mild and 10.8 ± 0.8 years (mean \pm SE, range 5-16) in moderate asthmatics. Atopy was detected in one healthy patient, as well as in nine mild and 15 moderate asthmatics. The number of subjects tested by different methods varied throughout the study, reductions in size of a sample

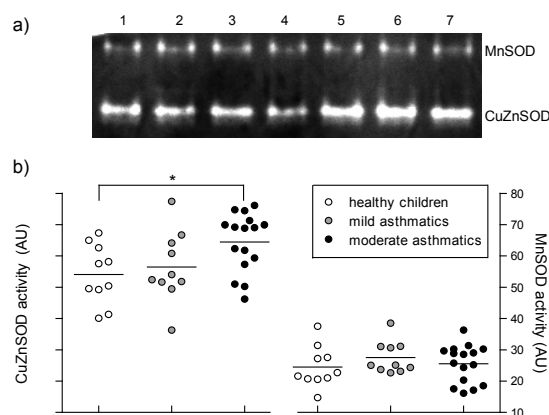


Fig. 1. MnSOD and CuZnSOD activity in PBMCs of healthy children and children with mild and moderate asthma. (A) A representative gel depicting SOD activities in PBMCs of two healthy children (lanes 1 and 2), two mild asthmatics (lanes 3 and 4), and three moderate asthmatics (lanes 5, 6, and 7). (B) Graphical presentation of individual data for CuZnSOD (left panel) and MnSOD (right panel) activities. Horizontal bars represent the mean values. *: $p < 0.05$

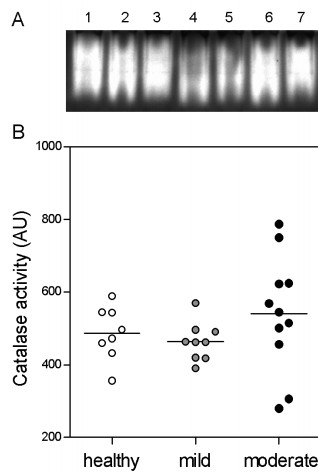


Fig. 2. CAT activity in PBMCs of healthy children and children with mild and moderate asthma. (A) A representative gel shows results obtained for two healthy children (lanes 1 and 2), two children with mild (lanes 3 and 4), and three with moderate asthma (lanes 5, 6, and 7). (B) Graphical presentation of the individual data. Horizontal bars represent the mean values.

being due to limited amounts of blood available and PBMC yields from certain patients.

To measure activity of the antioxidant enzymes

SOD and CAT, enzymatic gel assays were performed. Activities of both CuZnSOD and MnSOD were detected in PBMCs of all examined subjects ($n=36$). Quantitative analysis of SOD bands showed that CuZnSOD activity in PBMCs of children with moderate asthma was significantly higher ($p=0.01$) than in healthy children. The relative level of CuZnSOD activity found in children with mild asthma was lower compared to that in the group of moderate asthmatics, but the difference was not statistically significant. Similarly, there were no statistically significant differences of MnSOD activity between the three groups of patients (Fig. 1).

The results of CAT activity measurements in PBMCs of 20 asthmatic and eight healthy children are shown in Fig. 2. The level of CAT activity was found to be unaltered ($p=0.2$) in both groups of asthmatic patients in comparison to healthy children. The ratio of CuZnSOD to CAT activity, as an indicator of antioxidative enzyme (dis)balance, was significantly higher ($p=0.04$) in PBMCs of moderate asthmatic patients compared to healthy children, while differences between mild asthmatic and

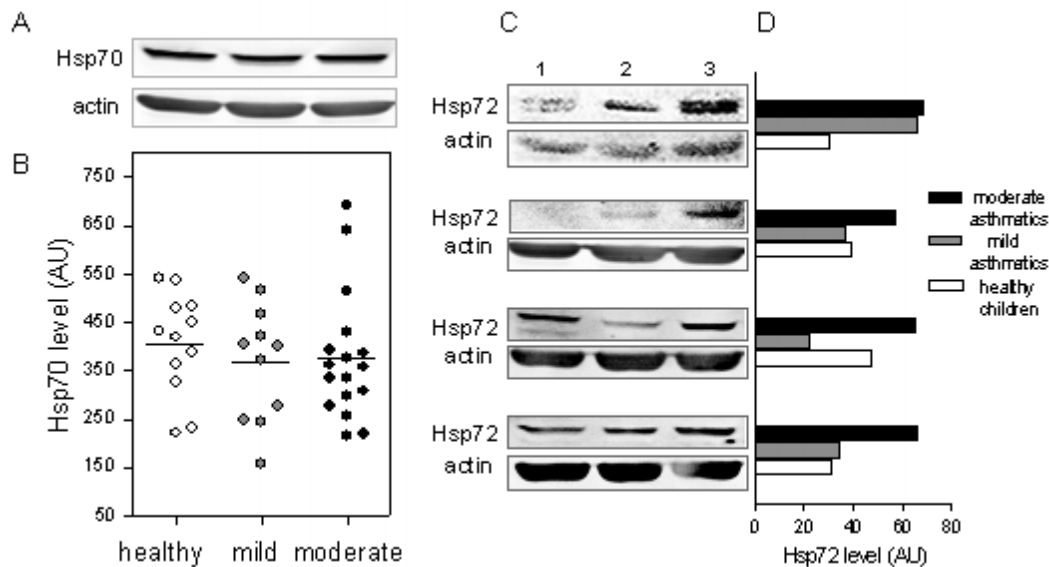


Fig. 3. Western blot analysis of Hsp70 expression in PBMCs of healthy children and children with mild and moderate asthma. (A) Western blot showing immunoreactive bands corresponding to both Hsp70 isoforms. (B) Individual data on expression of Hsp70. The representative bands are shown above the appropriate scattergrams. Horizontal bars represent the means. (C) Western blots showing Hsp72 expression in PBMCs isolated from four healthy children (lane 1), four mild asthmatics (lane 2), and four moderate asthmatics (lane 3). (D) Relative integrated optical density of Hsp72 bands.

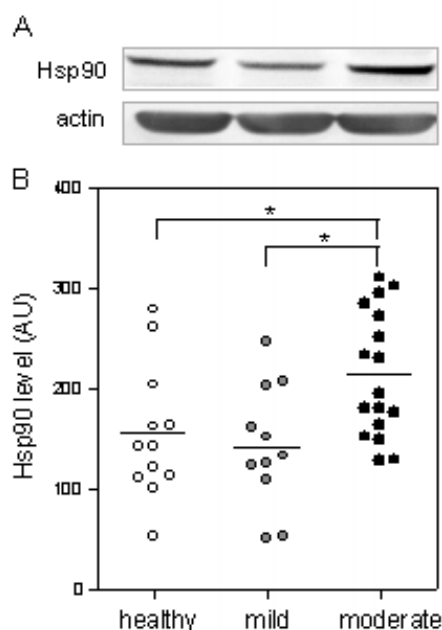


Fig. 4. Western blot analysis of Hsp90 in PBMCs of healthy children and children with mild and moderate asthma. (A) A representative Western blot showing Hsp90 expression. The representative bands are shown above the appropriate scattergrams. (B) Graphical presentation of the individual data. Horizontal bars represent the mean values. *, $p < 0.05$.

healthy children were not observed.

In order to examine the level of Hsp70 expression by Western blot analysis, two different monoclonal antibodies were applied: one recognizing both the constitutive (Hsc70) and the inducible (Hsp72) form of the protein, the other recognizing only Hsp72. Intensities of the immunoreactive bands corresponding to total Hsp70 (Hsc70+Hsp72) were similar ($p = 0.5$) in all studied groups of patients (Fig. 3A and B). However, the relative amount of the Hsp72 isoform was higher in moderate asthmatic patients than in mild asthmatics and healthy controls. Since the sample size for this analysis was rather small, with a total of four subjects per group, the results obtained for each individual patient are presented (Fig. 4C and D) and statistical analysis was not performed.

Western blot analysis revealed significant increase of Hsp90 expression in PBMCs of moderate asthmatics when compared to mild asthmatics ($p = 0.01$) and healthy children ($p = 0.01$) (Fig. 4).

DISCUSSION

The present study provides evidence indicating a disequilibrium in the activity of antioxidant enzymes in PBMCs of children with moderate asthma. This disequilibrium is manifested as an increase in the ratio of CuZnSOD to CAT activity in moderate asthmatics as compared to healthy children. To our knowledge, this is the first study examining relative activity of both intracellular SOD isoforms in childhood asthma. In PBMCs of moderate asthmatic patients, the activity of CuZnSOD was higher in comparison to healthy children, while that of MnSOD was similar in all three groups of subjects. The observed changes in antioxidant status are accompanied by elevated Hsp90 and Hsp72 expression.

The increase of SOD activity in moderate asthmatics, which can be ascribed solely to the cytoplasmic CuZnSOD isoform, is likely to reflect compartmentalized activity of the cell's antioxidant system. In keeping with the concept of "microdomain-specific signaling", which is based on spatial organization of oxidant production and antioxidant activity in the cell (Terada, 2006), increased activity of the CuZnSOD isoform parallel with unaltered MnSOD activity could indicate that the predominant source of superoxide anions in PBMCs of moderate asthmatic children resides in the cytoplasm rather than in mitochondria. Our results are in agreement with studies that showed increased superoxide production in asthmatic patients without addressing the source of the radical. For example, an increase in $O_2^{\cdot -}$ production from peripheral blood cells was registered in stable adult asthmatic patients (Vachier et al., 1994) and concentration of the superoxide anion was found to be elevated in the erythrocytes of symptom-free children with asthma (Shanmugasundaram et al., 2001).

Although evaluation of the serum antioxidant capacity in asthmatic children (Liao et al., 2004) and antioxidant status in red blood cells of asthmatic adults (Mak et al., 2004) showed an increase in SOD activity, recent findings suggest that SOD could be modulated by oxidative and nitrative reactive species and thus susceptible to inactivation, especially

by the powerful oxidant peroxynitrite (Quint et al., 2006; Alvarez et al., 2004). These mechanistic studies were extended by research on the molecular basis of asthma pathophysiology conducted by Comhair et al. (2005), who presented evidence for MnSOD inactivation in bronchial cells of asthmatic patients. The loss of MnSOD activity was correlated with physiological parameters of asthma *in vivo* and with apoptosis in airway epithelial cells. A direct correlation was also demonstrated between bronchial hyperreactivity in asthmatic patients and total SOD inactivation in BAL cells and bronchial brushing (Smith et al., 1997). On the contrary, we found increased activity of CuZnSOD in the group of children with more severe asthma, while the level of this enzyme's activity in PBMCs of mild asthmatic and healthy children was comparable. These discrepancies could be explained in several ways.

First of all, as opposed to the mentioned studies, the recruited asthmatic patients were classified into two homogeneous groups based on asthma severity, which enabled us to detect subtle differences of enzyme activity between different degrees of asthma severity. In the second place, the patients selected for our study were not treated with corticosteroids. Glucocorticoids are known to suppress the inflammatory response and have been shown to influence superoxide anion production. Superoxide release by mouse peritoneal macrophages *in vitro* was shown to be rapidly inhibited after glucocorticoid treatment (Long et al., 2005), as is the release by monocytes in treated asthmatic patients (Majori et al., 1998). We reasoned that enrolling stable and untreated patients would exclude the antioxidant and anti-inflammatory effects of this treatment on target molecules. Thus, the discrepancies might be attributable to differences of study design. Thirdly, there are no studies on the difference of SOD activity between asthmatic adults and children, but there may well be differences between childhood and adult asthma (Brown et al., 2005). Numerous studies have established the role of cytokine-mediated inflammation in the chronicity of asthma, and there is considerable evidence indicating that age is among the most important variables in cytokine expression (Krug et al., 1998). In addition, SODs

are induced and regulated by inflammatory cytokines (Marklund, 1992). Thus, the age of subjects enrolled in this study could be a confounding factor. Finally, based on the data available, the possibility of cell-specific differences in CuZnSOD and MnSOD activities cannot be excluded. Studies along these lines performed on pediatric subjects are scarce, being particularly limited by ethical and practical problems of performing invasive procedures of sample collection for research purposes only.

CAT activity is herein reported to be unaltered in asthmatic as compared to healthy children. Similarly, the activity of this enzyme did not differ in lungs (Smith et al., 1997) and erythrocytes (Pennings et al., 1999) of adult asthmatics as compared to the levels in healthy subjects. On the other hand, there are studies that reported a decrease of CAT activity in patients suffering from asthma. For example, a recent proteome survey identified an oxidative mechanism of CAT inactivation in asthmatic lungs (Gnosh et al., 2006). Novák et al. (1991) showed that blood catalase activity was decreased in stable asthmatic children. However, our finding should be considered in light of the previously discussed increase of CuZnSOD activity. Taken together, our results suggest that an increase of CuZnSOD activity that is not accompanied by concomitant increase of CAT activity could lead to an imbalance between H_2O_2 production and removal. As a consequence of the increased ratio of CuZnSOD to CAT activity in PBMCs of moderate asthmatics, elevated production of H_2O_2 could, hypothetically, be expected. A similar mechanism of H_2O_2 accumulation has been proposed to support the oxidative stress hypothesis of Alzheimer disease pathology (Gsell et al., 1995) and has been shown to contribute to cellular senescence in the aging process (de Haan et al., 1996). Most importantly, an increased H_2O_2 level has been measured in exhaled breath condensate of stable asthmatic children, particularly those not treated with glucocorticoids (Jobsis et al., 1997). Data indicating an imbalance in the functional complex of enzymes that are a potential source of H_2O_2 also raise a question as to the well-known ability of this oxidant to inhibit SOD activity. Taking into account that the patients included in this study were

asymptomatic, we assume that in the period prior to and during an acute asthma attack, the elevation of inflammatory mediators would cause additional increase of CuZnSOD activity and a consequent rise in the concentration of H_2O_2 , consecutively leading to a subsequent loss of enzyme activity. Such an assumption is supported by the study of *Comhair et al.* (2005), who reported rapid loss of SOD activity during an asthma attack experimentally induced in asthmatic patients.

It has been proposed that HSPs could also play a role in asthma. In line with this proposal, the expression of Hsp70 in airway cells of asthmatics was found to be elevated and correlated with disease severity (*Vignola et al.*, 1995). In our study, when the expression of Hsp70 was followed using an antibody that recognizes both the inducible (Hsp72) and the constitutive (Hsc70) isoform of the protein, differences between healthy and asthmatic children were not observed. However, although performed on a small number of subjects, examination of the level of the Hsp72 isoform provided evidence for higher abundance of this isoform in PBMCs from children suffering from a more severe form of the disease in comparison to healthy children and mild asthmatics. It is logical to assume that the level of the constitutive Hsc70 in PBMCs was high enough to mask changes in the level of the inducible member of the Hsp70 family. Importantly, *Polla et al.* (1996) demonstrated that Hsp70 prevented H_2O_2 -induced oxidative damage in mitochondria. Higher expression of Hsp72 in PBMCs may additionally support the hypothesis of *Ménoret et al.* (2002) that overexpression of this protein, under oxidative stress, can efficiently stimulate an immune response.

Elevated Hsp90 expression in moderately asthmatic children has also been observed. There are few data on the role and level of Hsp90 expression in asthma. Generally, heat shock protein expression is stimulated by increased concentrations of misfolded proteins, but their role in transmission of redox changes in the cell is currently a topic of active investigation. Several chaperones (such as prokaryotic Hsp33), having highly reactive cysteins, are subject to redox control and are readily activated

in response to an oxidizing insult (such as H_2O_2 exposure), thereby protecting cells from oxidative damage (*Jacob et al.*, 1999). Likewise, specific redox-regulated interactions of Hsp90's reactive cystein residues with cytochrome c suggest a role of this chaperone in the regulation of cellular functions under oxidative stress (*Nardai et al.*, 2000). Further studies along these lines are necessary in order to elucidate the possible role of Hsp90 overexpression in asthma.

Results of the present study add to the growing body of evidence demonstrating that the pathophysiology of asthma is associated with alterations in the level of antioxidants and oxidants. In our study, an imbalance in the activities of primary antioxidant enzymes was observed in PBMCs of children with moderate asthma. However, it is not possible to link this imbalance with severity of the disease, since no significant differences of antioxidant enzyme activities were found between moderate and mild asthmatics. On the contrary, the increase in the content of Hsp90 and possibly Hsp72 observed in moderate vs. mild asthmatics and healthy children suggests that alterations in the cellular stress response system might be related to severity of the disease. An obvious need exists for larger-scale studies on an even wider range of asthma severity in order to establish reliable association between antioxidant enzyme activities and HSP expression on the one hand, and lung function parameters and severity of asthma on the other. Such an association might eventually prove to be a valid criterion on which to base treatment modulation. Recent investigations have reported the beneficial effects of new classes of antioxidants in reducing the oxidative burden in chronic airway diseases (*Kirkham and Rahman*, 2006). They could be of particular relevance to pediatric patients, considering the adverse effects of long-term use of corticosteroids on growth, skeletal changes, and the endocrine system.

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REFERENCES

- Alvarez, B., Demicheli, V., Durán, R., Trujillo, M., Cerveñansky, C., Freeman, B. A., and R. Radi (2004). Inactivation of human Cu,Zn superoxide dismutase by peroxynitrite and formation of histidiny radical. *Free Radic. Biol. Med.* **37**, 813-822.
- Beauchamp, C., and I. Fridovich (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**, 276-287.
- Bertorelli, G., Bocchino, V., Zhuo, X., Chetta, A., Del Donno, M., Foresi, A., Testi, R., and D. Olivieri (1998). Heat shock protein 70 upregulation is related to HLA-DR expression in bronchial asthma. Effects of inhaled glucocorticoids. *Clin. Exp. Allergy* **28**, 551-560.
- Brown, V. G., and M. Ennis (2005). T cell cytokine production in childhood asthma. *Curr. Respir. Med. Rev.* **1**, 1-6.
- Calabrese, V., Butterfield, D. A., Scapagnini, G., Stella, A. M., and M. D. Maines (2006). Redox regulation of heat shock protein expression by signaling involving nitric oxide and carbon monoxide: relevance to brain aging, neurodegenerative disorders, and longevity. *Antioxid. Redox. Signal.* **8**, 444-477.
- Comhair, S. A., Xu, W., Ghosh, S., Thunnissen, F. B., Almasen, A., Calhoun, W. J., Janocha, A. J., Zheng, L., Hazen, S. L., and S. C. Erzurum (2005). Superoxide dismutase inactivation in pathophysiology of asthmatic airway remodeling and reactivity. *Am. J. Pathol.* **166**, 663-674.
- de Haan, J. B., Cristiano, F., Iannello, R., Bladier, C., Kelner, M. J., and I. Kola (1996). Elevation in the ratio of Cu/Zn-superoxide dismutase to glutathione peroxidase activity induces features of cellular senescence and this effect is mediated by hydrogen peroxide. *Hum. Mol. Genet.* **5**, 283-292.
- Gnosh, S., Janocha, A. J., Aronica, M. A., Swaidani, S., Comhair, S. A., Xu, W., Zheng, L., Kaveti, S., Kinter, M., Hazen, S. L., and S. C. Erzurum (2006). Nitrotyrosine proteome survey in asthma identifies oxidative mechanism of catalase inactivation. *J. Immunol.* **176**, 5587-5597.
- Gsell, W., Conrad, R., Hickethier, M., Sofic, E., Frolich, L., Wichart, I., Jellinger, K., Moll, G., Ransmayr, G., Beckmann, H., and P. Riederer (1995). Decreased catalase activity but unchanged superoxide dismutase activity in brains of patients with dementia of Alzheimer type. *J. Neurochem.* **64**, 1216-1223.
- Jakob, U., Muse, W., Eser, M., and J. C. Bardwell (1999). Chaperone activity with a redox switch. *Cell* **96**, 341-352.
- Jobsis, Q., Raatgeep, H. C., Hermans, P. W., and J. C. de Jongste (1997). Hydrogen peroxide in exhaled air is increased in stable asthmatic children. *Eur. Respir. J.* **10**, 519-521.
- Kinnula, V. L., and J. D. Carpo (2003). Superoxide dismutases in the lung and human lung diseases. *Am. J. Respir. Crit. Care. Med.* **167**, 1600-1619.
- Kirkham, P., and I. Rahman (2006). Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. *Pharmacol. Ther.* **111**, 476-494.
- Krug, N., Jung, T., Napp, U., Wagner, K., Schultze-Werninghaus, G., Heusser, C., Rieger, C. H., Schauer, U. and H. Fabel (1998). Frequencies of T cells expressing interleukin-4 and interleukin-5 in atopic asthmatic children. Comparison with atopic asthmatic adults. *Am. J. Respir. Crit. Care. Med.* **158**, 754-759.
- Liao, M. F., Chen, C. C., and M. H. Hsu (2004). Evaluation of the serum antioxidant status in asthmatic children. *Acta Paediatr. Taiwan* **45**, 213-217.
- Long, F., Wang, Y. X., Liu, L., Zhou, J., Cui, R. Y., and C. L. Jiang (2005). Rapid nongenomic inhibitory effects of glucocorticoids on phagocytosis and superoxide anion production by macrophages. *Steroids*, **70**, 55-61.
- Majori, M., Vachier, I., Godard, P., Farce, M., Bousquet, J., and P. Chanez (1998). Superoxide anion production by monocytes of corticosteroid-treated asthmatic patients. *Eur. Respir. J.* **11**, 133-138.
- Mak, J. C., Leung, H. C., Ho, S. P., Law, B. K., Lam, W. K., Tsang, K. W., Ip, M. S., and M. Chan-Yeung (2004). Systemic oxidative and antioxidative status in Chinese patients with asthma. *J. Allergy Clin. Immunol.* **114**, 260-264.
- Marklund, S. L. (1992). Regulation by cytokines of extracellular superoxide dismutase and other superoxide dismutase isoenzymes in fibroblasts. *J. Biol. Chem.* **267**, 6696-6701.
- Ménoret, A., Chaillot, D., Callahan, M., and C. Jacquin (2002). Hsp70, an immunological actor playing with the intracellular self under oxidative stress. *Int. J. Hyperthermia*, **18**, 490-505.
- Nardai, G., Sass, B., Eber, J., Orosz, G., and P. Csermely (2000). Reactive cysteines of the 90-kDa heat shock protein, Hsp90. *Arch. Biochem. Biophys.* **384**, 59-67.
- Novák, Z., Neméth, I., Gyurkovits, K., Varga, S. I., and B. Matkovics (1991). Examination of the role of oxygen free radicals in bronchial asthma in childhood. *Clin. Chim. Acta* **201**, 247-252.
- Peng, J., Jones, G. L., and K. Watson (2000). Stress proteins as biomarkers of oxidative stress: effects of antioxidant supplements. *Free Radic. Biol. Med.* **28**, 1598-1606.
- Pennings, H. J., Borm, P. J., Evelo, C. T., and E. F. Wouters (1999). Changes in levels of catalase and glutathione in erythrocytes of patients with stable asthma treated with beclomethasone dipropionate. *Eur. Respir. J.* **13**, 1260-1266.

- Pockley, A. G. (2003). Heat shock proteins as regulators of the immune response. *Lancet* **362**, 469-476.
- Polla, B. S., Bachelet, M., Dall'ava, J., and A. M. Vignola (1998). Heat shock proteins in inflammation and asthma: Dr Jekyll or Mr Hyde? *Clin. Exp. Allergy* **28**, 527-529.
- Polla, B. S., Kantengwa, S., Francois, D., Salvioli, S., Franceschi, C., Marsac, C., and A. Cossarizza (1996). Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *Proc. Natl. Acad. Sci. USA* **93**, 6458-6463.
- Quint, P., Reutzel, R., Mikulski, R., McKenna, R., and D. N. Silverman (2006). Crystal structure of nitrated human manganese superoxide dismutase: Mechanism of inactivation. *Free Radic. Biol. Med.* **40**, 453-458.
- Shanmugasundaram, K. R., Kumar, S. S., and S. Rajajee (2001). Excessive free radical production in the blood of children suffering from asthma. *Clin. Chim. Acta* **305**, 107-114.
- Smith, L. J., Shamsuddin, M., Sporn, P. H., Denenberg, M., and J. Anderson (1997). Reduced superoxide dismutase in lung cells of patients with asthma. *Free Radic. Biol. Med.* **22**, 1301-1307.
- Terada, L. S. (2006). Specificity in reactive oxidant signaling: think globally, act locally. *J. Cell Biol.* **174**, 615-623.
- Vachier, I., Chanez, P., Le Doucen, C., Damon, M., Descomps, B., and P. Godard (1994). Enhancement of reactive oxygen species formation in stable and unstable asthmatic patients. *Eur. Respir. J.* **7**, 1585-1592.
- Vignola, A. M., Chanez, P., Polla, B. S., Vic, P., Godard, P., and J. Bousquet (1995). Increased expression of heat shock protein 70 on airway cells in asthma and chronic bronchitis. *Am. J. Respir. Cell. Mol. Biol.* **13**, 683-691.

ПРОМЕНЕ АКТИВНОСТИ АНТИОКСИДАТИВНИХ ЕНЗИМА И КОНЦЕНТРАЦИЈЕ ПРОТЕИНА ТОПЛОТНОГ СТРЕСА У ЛИМФОЦИТИМА ДЕЦЕ ОБОЛЕЛЕ ОД АСТМЕ

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Циљ ове студије био је да се испита да ли су промене антиоксидативног статуса и експресије главних унутарћелијских стрес протеина повезане са патогенозом астме и тежином овог обољења код деце. Активност бакар/цинк (CuZn)- и манган (Mn)-супероксид дисмутаза (SOD), и каталазе (CAT) у мононуклеарним ћелијама из периферне крви 28 деце са стабилном астмом (11 са благим и 17 са средње тешким обликом болести) и 12 здраве деце испитана је ензимским тестом у нативном гелу. Концентрације два испитивана протеина тоplotног стреса, Hsp70 и Hsp90, одређене су техником квантитативног

имуноблота. Пацијенти са средње тешком астмом показали су повећану активност CuZn-SOD у поређењу са здравом децом, док разлике у активности Mn-SOD и CAT између испитиваних група пацијената нису запажене. Ниво Hsp90 и индуцибилне изоформе Hsp70 био је повећан код средње тешких астматичара у поређењу са благим астматичарима и здравом децом. Резултати указују да дисбаланс у ћелијском антиоксидативном и анти-стрес систему може допринети патофизиологији дечје астме, али не мора бити повезан са тежином обољења.